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(54) Title: METHOD OF CONTROLLING PLANT PATHOGENIC FUNGI

(57) Abstract

Ribonucleases from plants or microorganisms control fungal and nematode damage to plants. Genes encoding these proteins may be cloned into vectors for transformation of plant-colonizing microorganisms or plants, thereby providing a method of inhibiting fungal or nematode damage to plants.

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# METHOD OF CONTROLLING PLANT PATHOGENIC FUNGI

This application is a continuation-in-part of U.S. Serial Number 08/010,403, filed January 29, 1993.

## FIELD OF THE INVENTION

This invention relates to a method of controlling plant pathogenic fungi by a protein which may be applied directly to the plant or produced thereon by microorganisms or by genetically modifying the plant to produce the protein, and to genes, microorganisms, and plants useful in that method.

## BACKGROUND OF THE INVENTION

Ribonucleases are enzymes which cleave RNA by a variety of mechanisms. The enzyme may attack the terminal diester bond of the nucleic acid; it may hydrolyze the 3'-phosphate, or it may split the nucleotide chain without hydrolysis. There are various mechanisms, but the end result is a degradation of RNA. [Wilson]

Plant ribonucleases have been evaluated for their function in self-incompatibility, which is a mechanism to prevent inbreeding in plants. The enzymes are typically glycoproteins which operate as gametophytic or sporophytic incompatibility systems. [Haring, et al.] Until now no other practical function was known.

In the search for proteins which may impart fungal resistance to plants, many different types of antifungal proteins have been found. For example, chitinase functions in plants to ward off fungal infection. [Broglie et al.] Pathogenesis-related (PR) proteins have also been identified. For example, members of the PR-5 group of proteins, which are somewhat homologous to thaumatin, have been shown to rupture fungal hyphae by an unknown mechanism. European Patent Application 0 392 225 (Ciba-

Geigy AG) discloses PR proteins and transgenic plants containing genes encoding them; the plants are thus made more resistant to attack by fungi.

## SUMMARY OF THE INVENTION

It has been found that ribonucleases have antifungal properties. It is an object of the present invention to provide a ribonuclease from a plant or microorganism that is capable of reducing or eliminating the damage caused by plant fungal pathogens and genes useful in producing such proteins. It is a further object of the present invention to provide genetic constructs for and methods of inserting such genetic material into plant-colonizing microorganisms and plant cells. It is another object of the present invention to provide transformed microorganisms and plants containing such genetic material.

Additionally, the plants may also be transformed to co-express other antifungal proteins or insecticidal proteins, for example, using *Bacillus thurengiensis* (*B.t.*) genes. Examples of plants transformed to express *B.t.* genes are disclosed in European Patent Publication No. 0 385 962, which corresponds to U.S. Serial Number 07/476,661, filed February 12, 1990 [Fischhoff et al.], which is incorporated herein by reference. Another example of genes which may be coexpressed with the proteins of the present invention is the group of PR proteins having antifungal activity, as discussed above. Examples are found in EP 0 460 753 [Woloshuk] and copending U.S. Serial No. 953,495 (Bunkers et al.), which are osmotin-like proteins. An advantage of co-expressing other antifungal proteins lies in the use of more than one mode of action for controlling fungal damage which minimizes the possibility of the development of resistant fungal strains.

In accomplishing the foregoing, there is provided, in accordance with one aspect of the present invention, a method of controlling fungal damage to plants by providing a ribonuclease to the plant locus.

In accordance with another aspect of the present invention, there is provided a recombinant, double-stranded DNA molecule comprising in operative sequence:

a) a promoter which functions in plant cells to cause the production of an RNA sequence; and

b) a structural coding sequence that codes for production of a ribonuclease;

c) a 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence,

said promoter being heterologous with respect to the structural coding sequence.

In accordance with another aspect of the present invention, there is provided a method of producing genetically transformed plants which express an antifungal amount of a ribonuclease, comprising the steps of:

a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising

(i) a promoter which functions in plant cells to cause the production of an RNA sequence;

(ii) a structural coding sequence that codes for production of a ribonuclease;

(iii) a 3' non-translated region which functions in said plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence,

said promoter being heterologous with respect to the structural coding sequence;

b) obtaining transformed plant cells; and

c) regenerating from the transformed plant cells genetically transformed plants which express an fungal inhibitory amount of a ribonuclease.

There is also provided, in accordance with another aspect of the present invention, bacterial and transformed plant cells that contain DNA comprised of the above-mentioned elements (i), (ii), and (iii).

As used herein, the term "ribonuclease" is used to indicate an enzyme from plants or microorganisms which uses RNA as a substrate.

As used herein, the term "controlling fungal damage" is used to indicate causing a reduction in damage to a crop due to infection by a fungal pathogen.

As used herein, the term "structural coding sequence" means a DNA sequence which encodes for a polypeptide, which may be made by a cell following transcription of the DNA to mRNA, followed by translation to the desired polypeptide.

As used herein, the term "plant locus" means the area immediately surrounding a plant and including the plant and its root zone.

## DETAILED DESCRIPTION OF THE INVENTION

The method of the present invention may be carried out in a variety of ways. The antifungal protein, prepared by various techniques, may be directly applied to plants in a mixture with carriers or other additives, including other antifungal agents. The protein may be expressed by bacterial or yeast cells which have been applied to the plant. Preferably plant cells are transformed by one or more means to contain the gene encoding a ribonuclease which is expressed constitutively or in certain plant parts or upon exposure of the plant to the fungal infection.

One embodiment of the present invention comprises a protein isolated from the leaves of *Engelmannia pinnatifida*, commonly known as Engelman's daisy. This protein, designated P8, has been purified to homogeneity by ammonium sulfate precipitation, Mono-Q ion exchange and $C_{18}$ reverse phase column chromatography. The purified protein has a

molecular weight of almost 30.0 kD. It inhibits the growth of the agronomically important pathogens, *Phytophthora infestans* (*Pi*), a causal fungal pathogen of late blight disease in potato and tomato, and *Gaeumannomyces graminis* var *tritici* (*Ggt*), a causal fungal pathogen of Take-all disease in cereals, with an amount as little as 50 ng under the assay conditions. N-terminal amino acid sequence analysis of the purified P-8 protein indicates that it has some degree of homology to a known phosphate starvation-induced ribonuclease from tomato [Loeffler], but P-8 was isolated from a perennial plant not under any known extraordinary stress.

Another embodiment of the present invention comprises a ribonuclease derived from a microorganism. Examples are ribonucleases from *Bacillus cereus*, *E. coli*, and *Physarum polycephalum*. If plant expression of such proteins is desired, the genes may be extracted by known methods and inserted into the genome of plants, optionally after synthesizing new genes having improved expression levels *in planta*.

The plants that may be protected by the methods of the present invention will depend on the level of protection needed for the fungal pathogens of that plant type. For example, many vegetables such as potatoes and tomatoes may be protected from *Pi* by the present methods. However, other *Phytophthora* species are pathogenic to many other plants, such as fruit trees or turf, and thus these plants may also be protected by the methods of the present invention. Furthermore, wheat and barley plants may be protected from *Ggt* by the present method.

As noted above, the antifungal proteins of the present invention may be used in combination with other antifungal proteins so as to provide a broad spectrum of activity, i.e., control additional pathogens, and/or provide multiple modes of action for the inhibition of same fungal pathogen. Sources of such antifungal proteins might be plants, such as the proteins of the present invention, or may be microbial or other nonplant organisms.

## BIOEFFICACY ASSAYS

Antifungal assays with P8

Assays for activity against *Pi* and *Ggt* were conducted with P8 protein. The growth medium for the *Pi* and *Ggt* assays was made from 100 mL V8® vegetable juice, 2 g calcium carbonate, 15 g bacto agar, and 900 mL water. The calcium carbonate was added to the V8® juice; then the mixture was decanted and combined with the rest of the ingredients. The medium was then autoclaved for 30 minutes. All reagents used were of the highest grade commercially available.

Antifungal activity of the protein was determined using a hyphal extension-inhibition assay as described by Roberts and Selitrennikoff. Typically, fungal mycelium was harvested from actively growing fungus and placed in the center of a sterile Petri dish containing nutrient agar. After incubation of the dish at 20 °C for 48-72 hr to allow for mycelial growth in a symmetrically circular shape, sterile paper discs (Difco concentration disc, 1/4) were positioned on the agar approximately 1.5 cm from the mycelium. 35 µL of a Tris buffer solution (25 mM, pH 8.0) containing <1 µg of the protein was applied to each disc. The plate was incubated at 20 °C overnight. The antifungal activity was determined based on the zone of hyphal extension inhibition exhibited in the vicinity of the discs. P8 protein demonstrated inhibition of *Pi* and *Ggt* with as little as 50 ng of protein per disc. Activity is noted by the formation of a crescent shaped zone of inhibition at the edge of the mycelial growth that was approaching the disc.

Antifungal assays with Microbial Ribonucleases

Assays for activity against *Pi* and *Septoria nodorum (Sn)* were conducted with two microbial ribonucleases. Preparations of a ribonuclease from *Bacillus cereus* and one from *Physarum polycephalum* were obtained

from United States Biochemical Corporation (Cleveland, Ohio) and tested at a concentration equal to 100 units of activity in assays designed for a 96-well plate. One "unit" is sufficient enzyme activity to give a uniform partial digestion of 3 µg of RNA when incubated at 55 °C for 12 min in 6 µl of assay buffer.

Tests against *Pi* were conducted in Medium #303, prepared as follows: One liter contains 1 g $MgSO_4 \cdot 7H_2O$; 2 g $KH_2PO_4$; 0.5 g NaCl; 1 g $CaCO_3$; 1 ml $ZnSO_4 \cdot 7H_2O$ stock- 1 mg/ml; 1 ml $FeSO_4 \cdot 7H_2O$ stock - 1 mg/ml; 0.5 ml FeEDTA stock - 100 mM; 20 g Maltrin M-100; 20 g Casein; 5 g Yeast Extract; 5 g Glucose; 3.02 g Pipes 10 mM; pH adjusted to 6.5 and filter sterilized. *Pi* is seeded at 5 x $10^3$ sporangia per well and allowed to incubate at 18 °C for 24-48 hours. Assessment of growth is made by measuring the OD at 595 nm. The ribonuclease from *Bacillus cereus* provided 59% inhibition; the ribonuclease from *Physarum polycephalum* provided 55% inhibition.

Tests against *Sn* were conducted in CDAA .1% media prepared as follows: 35 g/l Difco Czapek Dox Broth, 1 g/l Proline, 500 mg/l Asparagine, 500 mg/l Cysteine, and 1 g/l Agar are autoclaved for 23 minutes, and filter-sterilized vitamins (1 ppm Thiamine and 1 ppm Biotin) are added. A seven day old sporulating culture of *Septoria nodorum* on YMA agar is used to make the spore suspension. A small amount (<1 ml) of CDAA media is dropped onto an area of the culture with pink spore masses oozing from the pycnidia. The spores are mixed with the CDAA media by repeatedly drawing up and expelling them from the pipetter. The concentrated suspension is added to the total volume of CDAA required for the test, adjusting spore concentration to 50,000 spores/ml. Assay incubation is at 24° C in darkness. The spore suspension is dispensed at 50 µl/well in a 96 well microtiter plate. These plates are then placed in an incubator (10hr/day light at 12 °C) for 24 hours prior to sample application. 50 µl of

sample is added to the 50 µl of inoculum (prepared 24 hours earlier) resulting in a total well volume of 100 µl/treated well/replicate treatment. Assay plates are incubated for 48 hours and the results are determined by reading optical density (OD) with a BioRad microtiter plate reader model 3550 at a single wavelength of 595 nm. An OD reading is made at time zero ($t_0$) which is made immediately after sample application, and an OD reading is made at 48 hours after sample application ($t_{48}$). Fungal growth estimate is determined by the difference in OD readings between $t_0$ and $t_{48}$ multiplied by a calculation value for fungal biomass. (The calculation value for fungal biomass is the relationship between fungal growth and optical density and was determined in separate experiments. The relationship between fungal growth and optical density was determined by growing fungi in 96 well microtiter plates, and harvesting the mycelium over time, at absorbance intervals of approximately 0.1 OD. The calculation value comes from the linear relationship between fungal biomass and OD for the specific fungus. It is the slope value obtained from the linear realtionship. The calculation value for *Sn* is 0.508. Then % inhibition is determined from the difference between the biomass of the treatments and the biomass of the controls. The ribonuclease from *Bacillus cereus* provided no inhibition at the concentration tested; the ribonuclease from *Physarum polycephalum* provided 53% inhibition.

An assay again *Pi* was conducted with ribonuclease 1 of *E. coli* using the method described above. The ribonuclease sample was purchased from Ambion Corp. (Austin, TX). Ribonuclease 1 provided 65 percent inhibition of *Pi* at 10 µg/ml of assay medium.

## PROTEIN IDENTIFICATION

The active protein P8 from *Engelmannia pinnatifida* was isolated, purified, partially sequenced, and identified as having homology to a plant

ribonuclease. Other plant derived ribonucleases may be isolated by similar techniques. Bacteria and other microorganisms which are known or found to produce ribonucleases may be cultured by known methods and the ribonuclease extracted from the culture medium.

Protein Isolation

All purification procedures were carried at 4 °C unless otherwise noted. Briefly, 300 g of the leaves of *Engelmannia pinnatifida* were homogenized in 500 mL of 10 mM sodium acetate buffer, pH 5.0 (Buffer A). The precipitate from the extract was discarded by centrifugation and solid ammonium sulfate was added to the supernatant to 80% of saturation. After stirring overnight, the precipitate was collected by centrifugation, resuspended in Buffer A and extensively dialyzed against 10 mM Tris-HCl buffer, pH 8.0 (Buffer B), using a 12 kDa dialysis membrane. The dialyzed solution was then concentrated to about 25 mL using an Amicon centripep concentrator with 10 kDa molecular weight cutoff. The concentrated solution was passed over a PD-10 column equilibrated with Buffer B to remove any remaining small molecular weight molecules. The high molecular weight fraction was then applied on a Mono-Q column which was equilibrated with Buffer B. Separated components were eluted from the column with a gradient of NaCl (0 to 1.0 M) in Buffer B at a flow rate of 1 mL per min using a Waters Associate HPLC system (model 510). Active fractions were pooled and further purified on a Vydac $C_{18}$ reversed phase column equilibrated with 0.1% TFA in $H_2O$ (Buffer C). After washing with Buffer C for 5 min, the column was eluted at a flow rate of 1 mL per min at 25°C with a linear gradient of 0 to 80% acetonitrile in Buffer C. All fractions were collected, concentrated to dryness using a Savant concentrator system, redissolved in 0.5 mL of Buffer A and assayed for antifungal activity. A fraction which eluted at about 60% acetonitrile

showed strong antifungal activity. SDS-PAGE showed that this fraction contained a homogeneous protein with a molecular weight of almost 30 kDa.

Amino Acid Sequences

Protein samples were hydrolyzed for 24 hrs in 6 N HCl at 110 °C *in vacuo* and analyzed on a Beckman 630 High Performance Amino Acid Analyzer. Automated Edman degradation was carried out on an Applied Biosystems model 470A Protein Sequenator. The respective PTH-amino acid derivatives were identified by reversed phase analysis in an on-line fashion employing an Applied Biosystems model 120 PTH Analyzer. N-terminal sequencing of the P8 protein identified 16 amino acids with undetermined amino acids at positions 1, 2, and 14 of the mature protein.

## GENETIC IDENTIFICATION

Cloning of the P8 cDNA

The full length P8 cDNA was isolated in two separate stages. A partial cDNA was obtained using Polymerase Chain Reaction (PCR) based protocols of mixed oligonucleotide primed amplification of cDNA (MOPAC) [Lee] and rapid amplification of cDNA ends (R.A.C.E.) [Frohman]. First strand cDNA was generated from *Engelmannia pinnatifida* leaf poly A+ RNA and served as the template for MOPAC reactions. Primers for P8 gene specific amplification were mixed oligonucleotide 17-mers (32 fold degenerate) made to amino acids at positions 4 through 9 of the mature protein (SEQ ID NO:1). A second nested mixed oligonucleotide 18-mer (512 fold degenerate) was made to amino acids at positions 6 through 11 of the mature protein to verify the MOPAC PCR product as P8 specific (SEQ ID NO:2). A 934 bp fragment was identified and subcloned into pUC118 vector [Vieira] at the SmaI site (blunt ligation) resulting in pMON8969. The partial cDNA was sequenced and the deduced 5' sequence matched the

N-terminal protein sequence obtained, specifically amino acid positions 9 through 16 (region independent of PCR primer).

The P8 gene was isolated from a genomic library, which was made using DNA isolated from leaf tissue of *Engelmannia pinnatifida*. The genomic library was constructed from genomic DNA partially digested with MboI ligated into BamHI site of the lambda EMBL3 vector. [Frischauf et al.] The library was screened using pMON8969 cDNA insert and a 5'-specific, 34-mer oligonucleotide probe (SEQ ID NO:3). A hybridizing clone designated lambda clone #25 was identified as possibly carrying the P8 gene. The P8 gene was localized to two AccI fragments, a 4.7 kb fragment encoding the 5' region and a 1.5 kb fragment encoding the 3' region. The 4.7 kb AccI fragment was subcloned and the 5' region of the P8 gene was sequenced. The 5' DNA sequence encoded a 33 amino acid N-terminal signal sequence that is removed during protein processing and is not present in the mature protein. The first two N-terminal amino acids of the mature protein were identified as glutamic acid and histidine.

The full length P8 cDNA was generated by PCR using a 5' gene specific primer from the start codon (ATG) of the signal peptide with nested BamHI and BglII restriction sites (SEQ ID NO:4) and a 3' gene specific primer after the stop codon (TGA) with nested EcoRI and HindIII restriction sites (SEQ ID NO:5).

The full length P8 cDNA PCR product was subcloned as a 822 bp BglII/EcoRI fragment into a previously constructed *E.coli* cassette vector containing an enhanced CaMV 35S promoter. The 3' nontranslated polyadenylation sequence of the ssRUBISCO E9 gene was also provided as the terminator. The vector also contained a multilinker site between the leader and the terminator sequences, NotI sites before and after the promoter and the terminator sequences, and an ampicillin resistance gene. The full length P8 cDNA was sequenced and thus determined to be that

shown in SEQ ID NO:10. The deduced translated protein sequence is shown as SEQ ID NO:11.

## GENETIC TRANSFORMATION

Mutagenesis of the partial P8 cDNA

For expression of a ribonuclease in heterologous systems, baculovirus or plant host, it was necessary to engineer a signal peptide to the N-terminus of the near full length mature protein (missing the first two amino acids), to target the protein to the vacuole or to the extracellular space, away from the cytoplasm where RNA is present. Two signal peptides were selected, one from an *Arabidopsis* thaumatin-like protein (ATLP) and one from a *Nicotiana alata* self incompatibility protein (NA2-2), a known ribonuclease. [McClure]

In the design of the synthetic DNA signal peptides, conservation of the amino acids surrounding the proteolytic cleavage site (where the signal peptide is connected to the mature protein) was followed, and potato preferred codons were used when possible.

To incorporate the ATLP synthetic signal peptide with the near full length P8 mature protein and mobilize it as a intact chimeric protein coding sequence, nested BamHI/BglII restriction sites were designed at the 5' end upstream of the ATG start of the ATLP synthetic signal peptide and a BsaAI restriction site at the 3' end of the synthetic signal peptide (SEQ ID NO:6). A DraI restriction site was engineered at the 5' end of the near full length P8 cDNA (SEQ ID NO:7). Blunt ligation of the BsaAI and DraI restriction sites inserted codons for Ala and Thr as the first two amino acids of the modified mature P8 protein. Ala and Thr are the first two amino acids found in the native ATLP mature protein and should conserve the context around the signal peptide proteolytic cleavage site. Nested EcoRI/HindIII restriction sites were engineered after the stop codon TGA of the near full length P8 cDNA (SEQ ID NO:5). These modifications allow

the intact chimeric protein gene to be mobilized as a cassette (a nested BamHI/BglII (5') - EcoRI/HindIII (3') restriction fragment of 795 bp) with minimal noncoding flanking sequences, into pUC119 [Vieira] to create pMON8989.

To incorporate NA2-2 synthetic signal peptide with the near full length P8 mature protein and mobilize it as a intact chimeric protein coding sequence, nested BamHI/BglII restriction sites were designed at the 5' end upstream of the ATG start codon of the NA2-2 synthetic signal peptide and a BstBI restriction site at the 3' end of the synthetic signal peptide (SEQ ID NO:8). A NarI restriction site was engineered at the 5' end of the near full length P8 cDNA (SEQ ID NO:9). Ligation of BstBI and NarI restriction sites inserts codons for Ala, Phe, Ala, and Thr as the first four amino acids of the modified mature P8 protein. Ala and Phe are the first two amino acids found in native NA2-2 mature protein and should conserve the context around the signal peptide proteolytic cleavage site. Nested EcoRI/HindIII restriction sites were engineered after the stop codon TGA of the near full length P8 cDNA (SEQ ID NO:5). These modifications allow the intact chimeric protein to be mobilized as a cassette, (nested BamHI/BglII (5') - EcoRI/HindIII (3') restriction fragment of 800 bp) with minimal noncoding flanking sequences, into pUC119 [Vieira] to create pMON8990.

Expression of P8 in Baculovirus

A BglII-EcoRI fragment containing the chimeric P8 gene was inserted into pVL1392, a vector for transfection of baculovirus with a heterologous gene. [Luckow] This cloning placed the chimeric P8 under the control of the polyhedrin promoter. The gene was detected in the transfected virus using dot blot analysis. The expressed protein was inhibitory to *Pi* in a hyphal extension plate assay.

Plant Gene Construction

The expression of a plant gene which exists in double-stranded DNA form involves transcription of messenger RNA (mRNA) from one strand of the DNA by RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated region which adds polyadenylate nucleotides to the 3' end of the RNA. Transcription of DNA into mRNA is regulated by a region of DNA usually referred to as the "promoter." The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA and to initiate the transcription of mRNA using one of the DNA strands as a template to make a corresponding strand of RNA.

A number of promoters which are active in plant cells have been described in the literature. These include the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*), the cauliflower mosaic virus (CaMV) 19S and 35S promoters, the Figwort Mosaic Virus (FMV) 35S promoter, and the light-inducible promoter from the small subunit of ribulose 1,5-bis-phos-phate carboxylase (ssRUBISCO, a very abundant plant polypeptide). All of these promoters have been used to create various types of DNA constructs which have been expressed in plants. U.S. Patent Number 5,034322 (Fraley et al., 1991), herein incorporated by reference, discloses such uses.

Alternatively, the promoters utilized in the double-stranded DNA molecules may be selected to confer specific expression of a ribonuclease protein in response to fungal infection. The infection of plants by fungal pathogens triggers the induction of a wide array of proteins, termed defense-related or pathogenesis-related (PR) proteins [Bowles; Bol et al.; Linthorst]. Such defense-related or PR genes may encode enzymes (such as phenylalanine ammonia lyase, chalcone synthase, 4-coumarate coA ligase, coumaric acid 4-hydroxylase) of phenylpropanoid metabolism,

proteins that modify plant cell wall (such as hydroxyproline-rich glycoproteins, glycine-rich proteins, peroxidases), enzymes (such as chitinases and glucanases) that degrade the fungal cell wall, thaumatin-like proteins, or proteins of as yet unknown function. The defense-related or PR genes have been isolated and characterized from a number of plant species. The promoters of these genes may be used to attain expression of ribonuclease in transgenic potato plants when challenged with *Pi*. Such promoters may derive from defense-related or PR genes isolated from potato itself [Fritzemeier et al.; Cuypers et al.; Logemann et al.; Matton and Brisson; Taylor et al.; Matton et al.; Schroder et al.]. In order to place the ribonuclease under the control of a promoter induced by infection with *P. infestans* the promoter reported by Taylor et al. may be preferred.

The particular promoter selected should be capable of causing sufficient expression of the enzyme coding sequence to result in the production of an effective amount of ribonuclease. A preferred promoter is a constitutive promoter such as FMV 35S or CaMV 35S.

The promoters used in the DNA constructs (i.e. chimeric plant genes) of the present invention may be modified, if desired, to affect their control characteristics. For example, the CaMV 35S promoter may be ligated to the portion of the ssRUBISCO gene that represses the expression of ssRUBISCO in the absence of light, to create a promoter which is active in leaves but not in roots. The resulting chimeric promoter may be used as described herein. For purposes of this description, the phrase "CaMV 35S" promoter thus includes variations of CaMV 35S promoter, e.g., promoters derived by means of ligation with operator regions, random or controlled mutagenesis, etc. Furthermore, the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression. Examples of such enhancer sequences have been reported by Kay et al.

An enhanced CaMV 35S promoter has been constructed as follows. A fragment of the CaMV 35S promoter extending between position -343 and +9 was previously constructed in pUC13. [Odell et al.] This segment contains a region identified as being necessary for maximal expression of the CaMV 35S promoter. It was excised as a ClaI-HindIII fragment, made blunt ended with DNA polymerase I (ClaI fragment) and inserted into the HincII site of pUC18. This upstream region of the 35S promoter was excised from this plasmid as a HindIII-EcoRV fragment (extending from -343 to -90) and inserted into the same plasmid between the HindIII and PstI sites. The enhanced CaMV 35S promoter (hereafter "CaMV E35S") thus contains a duplication of sequences between -343 and -90. [Kay et al.]

The RNA produced by a DNA construct of the present invention also contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNA's, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. Rather, the non-translated leader sequence can be derived from an unrelated promoter or coding sequence. For example, the petunia heat shock protein 70 (Hsp70) contains such a leader. [Winter]

As noted above, the 3' non-translated region of the chimeric plant genes of the present invention contains a polyadenylation signal which functions in plants to cause the addition of adenylate nucleotides to the 3' end of the RNA. Examples of preferred 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylate signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene and (2) plant genes like the soybean 7s storage protein genes and the pea ssRUBISCO E9 gene. [Fischoff, et al.]

Plant Transformation and Expression

An *E. coli* plasmid cassette vector, designated pMON8999, was preliminarily constructed for transformation of dicotyledonous plants. The BglII-EcoRI fragment containing the ATLP signal peptide-P8 protein coding sequence from pMON8989 was inserted into a multilinker cloning site of a previously constructed vector containing a CaMV E35S promoter. The 3' nontranslated polyadenylation sequence from pea ssRUBISCO E9 gene, was provided as the terminator downstream of the multilinker cloning site. The vector also contains NotI sites before the promoter and after the terminator sequences, and an ampicillin resistance gene.

The NotI-NotI 2.1 kb fragment from pMON8999, containing the E35S CaMV promoter, the ATLP-P8 gene, and the E9 3' terminator, was inserted into the NotI restriction site of pMON17227, a Ti plasmid vector disclosed and described by Barry et al. in WO 92/04449 (1991), incorporated herein by reference, to produce pMON22508. This vector contains the glyphosate resistance gene described by Barry for future selection of transformed plants.

A similar *E.coli* plasmid cassette vector, designated pMON22500, was constructed with the BglII-EcoRI fragment containing the NA2-2 signal peptide-P8 protein coding sequence inserted into a previously constructed vector pMON969 as described above. The NotI-NotI fragment from this resulting plasmid, containing the CaMV E35S promoter, the NA2-2-P8 gene, and the E9 3' terminator was inserted into the NotI site of pMON17227, described above, to produce pMON22509.

An additional *E.coli* plasmid cassette vector, designated pMON8992, was constructed by inserting the BglII-EcoRI fragment containing the NA2-2 signal peptide-P8 protein coding sequence into a previously constructed vector containing the FMV 35S promoter with a petunia Hsp70 leader sequence. The 3' nontranslated polyadenylation

sequence of the NOS gene was also provided as the terminator. The vector also contained a multilinker site between the leader and the terminator sequences, NotI restriction sites before and after the promoter and the terminator sequences, and an ampicillin resistance gene. The NotI-NotI 1.8 kb fragment from pMON8992, containing the FMV 35S promoter, the Hsp70 leader, the NA2-2 P8 gene, and the NOS 3' terminator, was inserted in both orientations into the NotI site of pMON17227, a Ti plasmid vector described above, to produce pMON22512 and pMON22513.

For the expression of the native full length P8 cDNA in plants, the NotI-NotI 2.17 kb fragment from pMON22518, containing the CaMV E35S promoter, the native full length P8 cDNA (SEQ ID NO:10), and the E9 3' terminator, was inserted in both orientations into the NotI site of pMON17227, a Ti plasmid vector described above, to produce pMON22519 and pMON22520.

Stable Transformation of Tobacco and Potato with a Ribonuclease Gene

Vectors pMON 22508, 22509, 22512, 22513, 22519 and 22520 were introduced into disarmed *Agrobacterium* ABI and used to transform potato explants in tissue culture. After selection for glyphosate resistance and plant regeneration, whole potato plants containing the ATLP-P8, the NA2-2-P8, and the native P8 coding sequences have been recovered and disease resistance assays have been performed. At least one transformation using pMON22509 and one using pMON22512 have resulted in potato plants which are more disease resistant than nontransformed or hollow vector control plants. The test was performed as follows:

Experiments were conducted using a randomized complete block design with 12 replicates ( three clonal plantlets per replicate ). Plantlets were inoculated with a suspension of $5 \times 10^4$ sporangia / ml to uniform wetness, and incubated at 19 °C. Plantlets were scored for late blight

development seven, eight, and nine days post inoculation, or until disease levels in the non transformed controls exceeded 80%. The amount of infected tissue was scored using a 0 - 9 rating scale, where 9 = 0% disease, 8 ≤ 10% disease, 7 = 11-25% disease, 6 = 26-40% disease, 5 = 41-60% disease, 4 = 61-70% disease, 3 = 71-80% disease, 2 = 81-90% disease, 1 = >90% disease.

The results of two independent experiments are given in Table 1.

Table 1

| | Disease Evaluation | |
|---|---|---|
| Line | Test 1 | Test 2 |
| 22509-4 | 3.18 | 4.50 |
| 22512-19 | 1.81 | 4.27 |
| 22509-5 | 1.91 | 3.50 |
| 22509-18 | 1.00 | 4.25 |
| Nontransgenic Control | 1.09 | 2.91 |
| Hollow vector Control | 1.63 | 2.40 |

Statistical analysis of the results indicates that line 22509-4 was improved over the controls in both tests. Lines 22512-19 and 22509-18 were improved over the control in test 2. Further testing continues.

Nematode Resistant Plants

Another use of the P8 protein is in the creation of nematode resistant plants. As described in International Patent Applications WO 92/21757 (Van der Eycken et al.) and WO 93/06710 (Conkling et al.), proteins which are toxic intracellularly, such as ribonucleases may be placed under the control of a nematode-induced promoter which is specific to the root cells on which nematodes feed. On infection, production of the ribonuclease is induced and the cell dies or is otherwise rendered unsuitable for nematodes to feed upon. Wild-type or mutated P8 would be useful in such a method of producing nematode resistant plants, particularly

potatoes. Thus a method of producing genetically transformed plants which are resistant to nematode damage would comprise the steps of:

a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising

(i) a promoter which functions in plant cells to cause the production of an RNA sequence in response to nematode attack of the root tissue;

(ii) a structural coding sequence that causes the production of a ribonuclease;

(iii) a 3' non-translated region which functions in said plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence;

b) obtaining transformed plant cells; and

c) regenerating from the transformed plant cells genetically transformed plants which express an amount of a ribonuclease effective to prevent the formation of nematode feeding sites.

All publications and patents mentioned in this specification are herein incorporated by reference as if each individual publication or patent was specifically and individually stated to be incorporated by reference.

From the foregoing, it will be seen that this invention is one well adapted to attain all the ends and objects hereinabove set forth together with advantages which are obvious and which are inherent to the invention. It will be understood that certain features and subcombinations are of utility and may be employed without reference to other features and subcombinations. This is contemplated by and is within the scope of the claims. Since many possible embodiments may be made of the invention without departing from the scope thereof, it is to be understood that all matter herein set forth is to be interpreted as illustrative and not in a limiting sense.

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SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Method of Controlling Plant Pathogenic Fungi

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/010403
(B) FILING DATE: 29-JAN-1993

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AARTTYGAYT TYTTYAC 17

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAYTTYTTYA CNCTNGCN 18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGGCCAGCA ACTTTCTGCT CAACGCATGA GAAC 34

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGATCCAGA TCTACAATGG CTTCACTCCC AACTAC 36

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCCAAGCTT GAATTCGAAA TGCATAATTG ATCAGAC 37

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGATCCAGA TCTATGGCTA ATCTTCTTGT TTCAACTTTT ATATTTAGTG CTCTTCTTTT 60

GATTTCAACT GCTACTGCTG CTACGTACCC C 91

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGTTTAAAG TTTGATTTTT TCACACTGGC TCTGCA 36

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGATCCAGA TCTATGTCAA AATCACAACT TACTTCAGTT TTCTTTATTC TTCTTTGTGC 60

TCTTAGTCCA ATTTATGGAG CTTTCGAATA TGGCATGCCC 100

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCGGCGCCA CTAAGTTTGA TTTTTTCACA CTGGCT 36

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 822 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 10..801

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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AGATCTACA ATG GCT TCA CTC CCA ACT ACT GTG ATC CTC CTA TCA GTT          48
          Met Ala Ser Leu Pro Thr Thr Val Ile Leu Leu Ser Val
            1               5                  10

CTG CTT ACC GGA TTG TTC TCC GTT GAT GGG AGA GCA GTT GAT CTC GTT         96
Leu Leu Thr Gly Leu Phe Ser Val Asp Gly Arg Ala Val Asp Leu Val
     15                  20                  25

TCA ACT CTC ACC GAA CAC ACA AAG TTT GAT TTC TTC ACA CTG GCT CTG        144
Ser Thr Leu Thr Glu His Thr Lys Phe Asp Phe Phe Thr Leu Ala Leu
 30                  35                  40                  45

CAA TGG CCA GCA ACT TTC TGC TCA ACG CAT GAG AAC AAA TGC TGT CCT        192
Gln Trp Pro Ala Thr Phe Cys Ser Thr His Glu Asn Lys Cys Cys Pro
                 50                  55                  60

GAA AAT GGT TGT TGC CAA GGA GGA AAT TCT CCA CCA GGA TTT ACA ATC        240
Glu Asn Gly Cys Cys Gln Gly Gly Asn Ser Pro Pro Gly Phe Thr Ile
             65                  70                  75

CAC GGA CTA TGG CCC GAT TAC AGT GAT GGA ACA TGG CCA TCA TGT TGT        288
His Gly Leu Trp Pro Asp Tyr Ser Asp Gly Thr Trp Pro Ser Cys Cys
         80                  85                  90

CCA GGT TCT GCT TAT GAT GAA ACA AAG ATT TCA CCA TTG CTT GAT GCT        336
Pro Gly Ser Ala Tyr Asp Glu Thr Lys Ile Ser Pro Leu Leu Asp Ala
     95                  100                 105

TTG CAC ACA TAT ATG CCG ATA CTA AGT TGT AAC GGT ATA GAA TCA TGC        384
Leu His Thr Tyr Met Pro Ile Leu Ser Cys Asn Gly Ile Glu Ser Cys
110                 115                 120                 125

GAT AAC AAG ACA AAG TCG TTT TGG GCT TAT CAA TGG GAA CAA CAT GGT        432
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Asp Asn Lys Thr Lys Ser Phe Trp Ala Tyr Gln Trp Glu Gln His Gly
                130                 135                 140

ACA TGT TCG ACC TTA GTA ACA GGC GAC CAA TAT GGA TAT TTC TTG ACA      480
Thr Cys Ser Thr Leu Val Thr Gly Asp Gln Tyr Gly Tyr Phe Leu Thr
            145                 150                 155

AGC CTT GAA TTG TAT TTG GAG TAT AAT GTG ACG GAA GTT CTA TCA AAA      528
Ser Leu Glu Leu Tyr Leu Glu Tyr Asn Val Thr Glu Val Leu Ser Lys
        160                 165                 170

GCT GGA ATT AAA CCT TCA AAT TCA AAG ACT TAC TCA TCT GCT GCC ATT      576
Ala Gly Ile Lys Pro Ser Asn Ser Lys Thr Tyr Ser Ser Ala Ala Ile
    175                 180                 185

GTT TCT ACT ATT GAA ACT GCT TTC GGT GCA CCA CCG CAA TTG GTC TGC      624
Val Ser Thr Ile Glu Thr Ala Phe Gly Ala Pro Pro Gln Leu Val Cys
190                 195                 200                 205

AAA AAT GGT GAT GTA ATT AAA GAA GTC CGG TTG TGT TTT ACC AAG CAC      672
Lys Asn Gly Asp Val Ile Lys Glu Val Arg Leu Cys Phe Thr Lys His
                210                 215                 220

CAC AAG ATC CGA GAG TGT GTG GAA CCT AGT TGG TGC CCT GAG TAT GTC      720
His Lys Ile Arg Glu Cys Val Glu Pro Ser Trp Cys Pro Glu Tyr Val
            225                 230                 235

AAG TTA CCA AAG TTT GCA TTC TTG GAT GGC ACG TCA AAG ACC GAG GAT      768
Lys Leu Pro Lys Phe Ala Phe Leu Asp Gly Thr Ser Lys Thr Glu Asp
        240                 245                 250

TTG CTG ATG TCT GCT ATT CGT TCA GTT GTC TGATCAATTA TGCATTTCGA        818
Leu Leu Met Ser Ala Ile Arg Ser Val Val
    255                 260

ATTC                                                                822
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 263 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Met Ala Ser Leu Pro Thr Thr Val Ile Leu Leu Ser Val Leu Leu Thr
 1               5                  10                  15

Gly Leu Phe Ser Val Asp Gly Arg Ala Val Asp Leu Val Ser Thr Leu
            20                  25                  30

Thr Glu His Thr Lys Phe Asp Phe Phe Thr Leu Ala Leu Gln Trp Pro
        35                  40                  45

Ala Thr Phe Cys Ser Thr His Glu Asn Lys Cys Cys Pro Glu Asn Gly
    50                  55                  60

Cys Cys Gln Gly Gly Asn Ser Pro Pro Gly Phe Thr Ile His Gly Leu
65                  70                  75                  80

Trp Pro Asp Tyr Ser Asp Gly Thr Trp Pro Ser Cys Cys Pro Gly Ser
                85                  90                  95

Ala Tyr Asp Glu Thr Lys Ile Ser Pro Leu Leu Asp Ala Leu His Thr
            100                 105                 110

Tyr Met Pro Ile Leu Ser Cys Asn Gly Ile Glu Ser Cys Asp Asn Lys
        115                 120                 125

Thr Lys Ser Phe Trp Ala Tyr Gln Trp Glu Gln His Gly Thr Cys Ser
    130                 135                 140

Thr Leu Val Thr Gly Asp Gln Tyr Gly Tyr Phe Leu Thr Ser Leu Glu
145                 150                 155                 160

Leu Tyr Leu Glu Tyr Asn Val Thr Glu Val Leu Ser Lys Ala Gly Ile
                165                 170                 175

Lys Pro Ser Asn Ser Lys Thr Tyr Ser Ser Ala Ala Ile Val Ser Thr
            180                 185                 190

Ile Glu Thr Ala Phe Gly Ala Pro Pro Gln Leu Val Cys Lys Asn Gly
        195                 200                 205
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Asp Val Ile Lys Glu Val Arg Leu Cys Phe Thr Lys His His Lys Ile
    210                 215                 220

Arg Glu Cys Val Glu Pro Ser Trp Cys Pro Glu Tyr Val Lys Leu Pro
225                 230                 235                 240

Lys Phe Ala Phe Leu Asp Gly Thr Ser Lys Thr Glu Asp Leu Leu Met
                245                 250                 255

Ser Ala Ile Arg Ser Val Val
            260
```

WHAT IS CLAIMED IS:

1. A method of controlling fungal damage to plants comprising providing to the plant locus a ribonuclease which inhibits fungal growth.

2. The method of Claim 1 wherein the fungus is *Phytophthora* sp. or *Gaeumannomyces* sp.

3. The method of Claim 1 wherein said protein is provided by plant-colonizing microorganisms which produce a ribonuclease which inhibits fungal growth.

4. The method of Claim 1 wherein said protein is provided by expression of a gene for the protein incorporated in the plant by previous genetic transformation of a parent cell of the plant.

5. The method of Claim 1 wherein said protein has the amino acid sequence SEQ ID NO:11.

6. The method of Claim 1 wherein said protein is P8.

7. A gene for a ribonuclease comprising SEQ ID NO:10.

8. A recombinant, double-stranded DNA molecule comprising in operative sequence:

a) a promoter which functions in plant cells to cause the production of an RNA sequence;

b) a structural coding sequence that encodes for production of a ribonuclease which inhibits fungal infection; and

c) a 3' non-translated region which functions in plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence,

said promoter being heterologous with respect to the structural coding sequence.

9. The DNA molecule of Claim 8 wherein said structural DNA sequence is SEQ ID NO:10.

10. The DNA molecule of Claim 8 wherein said promoter is selected from FMV35S and CaMV35S promoters.

11. A method of producing genetically transformed plants which express a fungal inhibitory-effective amount of a ribonuclease, comprising the steps of:

a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising

(i) a promoter which functions in plant cells to cause the production of an RNA sequence;

(ii) a structural coding sequence that causes the production of a ribonuclease;

(iii) a 3' non-translated region which functions in said plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence,

said promoter being heterologous with respect to the structural coding sequence;

b) obtaining transformed plant cells; and

c) regenerating from the transformed plant cells genetically transformed plants which express a fungal inhibitory-effective amount of a ribonuclease.

12. The method of Claim 11 wherein said structural DNA sequence is SEQ ID NO:10.

13. The method of Claim 11 wherein said promoter is selected from FMV35S and CaMV35S promoters.

14. A plant produced by the method of Claim 11.

15. The plant of Claim 14 wherein additional antifungal protein genes have been inserted into the genome of the plant.

16. The plant of Claim 14 also containing one or more genes expressing *B.t.* endotoxins.

17. The plant of Claim 14 which is a potato plant.

18. A potato seedpiece produced by a plant of Claim 17.

19. A method of producing genetically transformed plants which are resistant to nematode damage comprising the steps of:

a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising

(i) a promoter which functions in plant cells to cause the production of an RNA sequence in response to nematode attack of the root tissue;

(ii) a structural coding sequence that causes the production of a ribonuclease;

(iii) a 3' non-translated region which functions in said plant cells to cause the addition of polyadenylate nucleotides to the 3' end of the RNA sequence;

b) obtaining transformed plant cells; and

c) regenerating from the transformed plant cells genetically transformed plants which express an amount of a ribonuclease effective to prevent the formation of nematode feeding sites.